Examining the molecular interaction between potato (*Solanum tuberosum*) and Colorado potato beetle *Leptinotarsa decemlineata*

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Abstract: Colorado potato beetle (CPB) is a devastating herbivorous pest of solanaceous plants. Despite the economic impact, little is known about the molecular interaction of CPB with these plants. Using an 11 421 expressed sequence tag (EST) potato microarray, we identified 320 genes differentially expressed in potato leaves in response to CPB herbivory. Amongst these were genes putatively encoding proteinase inhibitors along with enzymes of terpenoid, alkaloid, and phenylpropanoid biosynthetic pathways, suggesting the defensive chemistries that constitute potato's defense against CPB herbivory. Several genes, such as those encoding proteinase inhibitors, represent mechanisms implicated in other plant–herbivory interactions, and could correspond with general defensive chemistry strategies. In other cases, products of the differentially expressed genes may represent taxa-specific defensive chemistry. For example, the presumed alkaloid products of a putative tropinone reductase I are specific to a subset of the Solanaceae. Two herbivory-induced genes, not specific to potato, are implicated in the synthesis of volatiles known to attract CPB predators. Comparison of continuous herbivore attack versus recovery from CPB attack indicates that fewer genes involved in defensive chemistry are induced after continuous feeding than after feeding and recovery, suggesting the plant's ability to mount a full defense response is enhanced under light versus heavy attack.

Key words: Colorado potato beetle, herbivore, infestation, plant–insect interaction, potato.

Résumé : La bête à patate du Colorado (CPB) constitue une peste herbivore dévastatrice chez les Solenaceae. En dépit de ses impacts économiques, on connaît peu de choses sur les interactions moléculaires entre le CPB et ces plantes. En utilisant un microarray 11 421 EST de la pomme de terre, les auteurs ont identifié 320 gènes à expression différentielle dans des feuilles de pommes de terre, en réaction à l'herbivorie par le CPB. Parmi ceux-ci, on retrouve des gènes putatifs codant pour des inhibiteurs de protéinases ainsi que des enzymes des sentiers biosynthétiques de terpénoïdes, d'alcaloïdes et de phénylpropanoïdes, signalant la chimie défensive constituant la défense de la pomme de terre contre l'herbivorie par le CPB. Plusieurs gènes, comme ceux codant des inhibiteurs de protéines, représentent des mécanismes impliqués dans d'autres réactions plante-herbivore, et pourraient correspondre à des stratégies générales de défenses chimiques. Dans d'autres cas, les produits de l'expression génétique différentielle peuvent représenter une chimie défensive spécifique au taxon. Par exemple, les produits alcaloïdes présumés d'une réductase I de la tropinone sont spécifiques à un sous-ensemble de Solenaceae. Deux gènes induits par l'herbivorie, non spécifiques à la pomme de terre, sont impliqués dans la synthèse de substances volatiles, reconnues pour attirer les prédateurs des CPB. La comparaison d'une attaque continue vs le rétablissement d'une attaque par le CPB indique que moins de gènes impliqués dans la chimie défensive sont induits après une attaque soutenue qu'après une attaque avec rétablissement, ce qui suggère que la capacité de la plante à mettre en branle une réaction de défense complète est intensifiée après une attaque courte vs une attaque prolongée.

Mots-clés : bête à patate du Colorado, herbivore, infestation, interaction plante–insecte, pomme de terre.

[Traduit par la Rédaction]

Introduction

Plants respond to feeding insects through a complex interaction involving the recognition of signals induced by mechanical wounding, as well as the detection of specific elicitors produced in either the regurgitant or salivary gland secretions of the insect. Broadly characterized as chewing, sucking, or mesophyll feeders, different types of insects are known to induce a different subset of plant responsive genes. In general, chewing insects such as *Manduca sexta* L. most closely mimic a mechanical wound response, inducing the production of jasmonic acid (JA) and the synthesis of JA responsive genes (McCloud and Baldwin 1997; Hermsmeier et al. 2001). Phloem feeding or sucking insects such as the aphids *Myzus persicae* (Sulzer) and *Brevicoryne brassicae* L., result in the induction of salicylic acid (SA) responsive genes, mimicking the response of plants to patho-

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gens (for review see De Vos et al. 2007). Whether this induction is a result of the presence of pathogens being vectored by the phloem-feeding insect is unclear. Generally, phloem-feeding insects produce little mechanical damage, which could explain why JA responsive genes are not as affected. Finally, cell content or mesophyll-feeding insects such as spider mites, *Tetranychus urticae* Koch, induce a combination of JA, ethylene (ET), and SA responsive genes (Kant et al. 2004).

In *Nicotiana attenuata* Torr. ex S. Watson, SA is produced upon infestation by all three types of insect pests, while JA is increased by only the chewing insect *Manduca sexta* (Heidel and Baldwin 2004). Using a whole genome microarray, De Vos et al. (2005) looked at the effect of insects from different feeding guilds on *Arabidopsis*. JA levels are induced by the chewing insect (*Pieris rapae* L.) and the cell content feeding thrip (*Frankliniella occidentalis* (Pergande)). More than 50% overlap between induced genes is found for these two feeding types, while little overlap is seen for the phloem feeder (*Myzus persicae*). This suggests that numerous genes are specifically expressed depending on the insect feeding type. Interestingly, the largest numbers of differentially expressed genes are found with the phloem feeder, which produces little phenotypic change upon infestation.

Insects can also be subdivided into generalists, those feeding on a number of plant species, and specialists, which are devoted to one or a few similar plant types. Almost identical transcript profiles were found in *Arabidopsis* when infested by the chewing insects, *Spodoptera littoralis* (Boisduval) (a generalist) and, *Pieris rapae* (a specialist), (Reymond et al. 2004). This indicates that the induced response does not distinguish between these two types of chewing insects. Later studies using *Arabidopsis* mutants for JA, SA, ET, or abscisic acid (ABA) signaling, revealed that there was a subtle difference in the plant response to these two insects. While both the specialist and generalist induce JA in the wild-type plant, in the JA mutant, *coi*1-1, *P. rapae* induces a distinct subset of genes (Bodenhausen and Reymond 2007) suggesting that this specialist may somehow suppress this subset of genes when JA is present. The SA and ET mutants, *npr*1-1, *sid*2-1, and *ein*2-1, however, have no such change. In terms of larval weight, both the generalist and the specialist insect gain more weight feeding on the JA mutant. The generalist insect *S. littoralis* gains less weight on SA and ET mutants compared with wild-type *Arabidopsis*, suggesting that these pathways negatively control insect resistance. Indeed, the transcript profile is also altered in the SA and ET mutants compared with wild-type *Arabidopsis* when fed upon by *S. littoralis*. Transcript profiles in ABA mutants are similar upon exposure to either generalist or specialist insect pests. It was found however that larvae of the generalist insect gain more weight on the ABA mutant compared with wild type. Clearly, JA plays the major role in defense against these insect pests in *Arabidopsis,* while the difference between feeding by a specialist versus a generalist may be the lack of induction of several genes in response to the specialist insect by the presence of JA.

Although chewing insects induce JA responsive genes and JA is also induced by mechanical damage, differences exist in the response of the plant to mechanical damage and chewing insects. For example, the volatile compounds, produced upon wounding often differ from those produced by herbivory (reviewed in Pare and Tumlinson 1999; Kessler and Baldwin 2002). This distinction allows predator or parasitoid insects to be attracted specifically to their prey on the infested plants. To distinguish whether these differences might be due to the magnitude and or timing of insect feeding, a mechanical worm has been constructed (mecworm) to mimic real insects. Damage caused by real insects and the mecworm is similar and induces a similar plant response (Mithofer et al. 2005). This suggests that the comparison of the response to herbivory and wounding may only be a matter of magnitude or timing.

However, when elicitors produced by the salivary glands or in the midgut are added to wounded leaves, it induces a response similar to the response by insect feeding. In fact, feeding by the caterpillar *Helicoverpa* reduces levels of the defense compound nicotine in *Nicotiana tabacum* L. This loss of nicotine can be reversed by ablation of the spinnerets, which are the primary secretory structures of the salivary glands in the caterpillar (Musser et al. 2002). In other words, the defense response to intact and ablated caterpillar spinnerets differs. This suggests that the elicitors such as glucose oxidase (Musser et al. 2002), or fatty acid amino acid conjugates (FACs) such as volicitin (Pohnert et al. 1999), or a small peptide derived from proteolysis of the plant derived enzyme cATP synthase such as inceptin (Schmelz et al. 2006), affect the wound response. Considering that inceptin was isolated from *Spodoptera frugiperda* (J.E. Smith) feeding on cowpea, while volicitin was isolated from the same genus *Spodoptera exigua* (Hübner) feeding on corn, this may explain in part the specificity of the plant–insect interaction.

In the current study, we used transcript profiling by microarrays to examine the interaction of Colorado potato beetle (CPB; *Leptinotarsa decemlineata* (Say), Coleoptera: Chrysomelidae) on potato (*Solanum tuberosum* L.). CPB is a specialist on potato resulting in hundreds of millions of dollars of crop losses annually in the US. Despite the economic importance of this pest, little is known about the molecular response of the plant to this chewing insect. Here, we examine this interaction by using an 11 421 expressed sequence tag (EST) microarray to identify the genes that are differentially expressed in potato in response to CPB feeding. Analyses of this data set suggest that chemical defenses may be a key part of the strategy that potatoes evoke as protection against herbivores, but that this defense may be affected by the intensity and (or) duration of the infestation.

Materials and methods

Plant material

Potato tubers from *Solanum tuberosum* 'Kennebec' were planted in individual 4 in. pots (1 in. = 25.4 mm) containing Metro-Mix® (Scotts Miracle-Gro Co., Marysville, Ohio). Plants were grown for 4 weeks during the winter season in a naturally lighted greenhouse without supplemental fertilization, and only plants with at least eight leaves were used in the tests. For real time quantitative reverse transcriptase polymerase chain reaction (RT-PCR) experiments, plants

were grown for 4 weeks without fertilization in a Conviron PGR15 growth chamber (Winnipeg, Manitoba) at 50% humidity with a 16 h light : 8 h dark cycle and 25 °C during the light phase (340 μmol·m^{-2}·s^{-1} at pot level) and 20 °C during the dark phase.

Colorado potato beetle rearing and infestation

CPB larvae were reared on *Solanum tuberosum* 'Kennebec'. For microarray experiments, experiments were conducted to compare control uninfested plants with plants that were either infested with CPB larvae for 1 h followed by 3 h of recovery, or that were infested with CPB larvae for 4 h continuously as indicated below. For the infestations, third- to fourth-instar CPB larvae were starved for 4 h, the eighth leaf from the bottom was covered with a fine mesh sleeve, and 10 larvae were added. Plants were divided into two groups: in group 1, CPB larvae were allowed to feed for 1 h and then removed, and the plants were harvested 3 h later, while in group 2, the leaf was continuously infested with 10 CPB larvae for 4 h and then harvested. Each treatment consisted of five plants arranged in a block design, and the five plants were pooled at the point of harvest to make up a single biological replicate. The experiment was then repeated a total of three times to generate three independent biological replicates. The position of each treatment block within the growth chamber was randomized amongst the three independent experiments.

For real time quantitative RT-PCR, time course experiments were performed in which control uninfested plants were compared with plants infested for 1 h followed by recovery for the specified time, or with plants infested continuously for the specified time. Plants were sampled at 2, 4, 7, or 23 h. For the controls, the fifth leaf from the bottom was harvested at the times indicated; with another set of plants, the fifth leaf from the bottom was enclosed in a fine mesh bag and 10 third- to fourth-instar unstarved CPB larvae were allowed to feed for 1 h and then removed, and the infested leaves were harvested at the times indicated; and, for the third group, entire plants were enclosed in a fine mesh bag, 10 unstarved CPB larvae were allowed to feed on the plants for the time indicated, and only the infested leaves of these plants were harvested. Zero time control leaves were harvested after the initial 1 h of infestation and were used to calculate a relative transcript level, with a value of 1 RQ (relative quantitation) being equal to the 0 h control. After that, the controls were harvested at the same time as the infested plants. For these experiments, the plants were divided into groups of two plants each, which were arranged in blocks according to the time variable. Pairs of plants were treated, and the harvested leaves from each pair were pooled at the point of harvest to make a single biological replicate. Three independent experiments were executed to generate three independent biological replicates, ensuring that the positions of both time and infestation treatment blocks within the growth chamber were randomized for each independent experiment.

Ribonucleic acid isolation for microarray and real time reverse transcriptase polymerase chain reaction

For microarray analyses, RNA was isolated from leaves with QIAGEN's RNeasy kit using the protocol recommended by the manufacturer (QIAGEN, Valencia, Calif.). The protocol is available at www.tigr.org/tdb/potato/microarray_SOPs.shtml. For real time quantitative RT-PCR, RNA was isolated using QIAGEN's RNeasy Plant Mini kit adding a RNase free DNase step using the manufacturer's protocol (QIAGEN).

Microarray

The TIGR potato 10K EST microarray contains 11 412 annotated cDNA clones spotted as duplicates on the array. The TIGR Solanaceae Expression Profiling Service performed all the microarray procedures including cDNA labeling, hybridization, data quantification, and data normalization using LOWESS. Protocols are available at www.tigr.org/tdb/potato/microarray_SOPs.shtml. For each treatment–control comparison, three biological replicates were analyzed; for each biological replicate, a dye-swap of technical replicates was performed. In total, 12 arrays for 1 and 4 h infestation were carried out. The data from the microarray experiments are available from the TIGR Solanaceae Gene Expression Database (www.tigr.org/tigr-scripts/tdb/potato/study/potato_study_hybs.pl?study=86&user=&pass=&sort=id&order=asc).

Exported data were analyzed in R (Ihaka and Gentleman 1996) using the BioConductor suite of packages (Gentleman et al. 2004). Quality assessment of the raw and background-corrected data was carried out by inspection of ratio-intensity plots (also known as minus–add (MA) plots), pairwise correlations of ratio (M) values between slides, and distribution and density of intensity (A) values. Data were analyzed with the linear models for microarray data (LIMMA) package (Smyth 2005) and exploratory analysis for two-color spotted micorarray data (marray) package (Yang and Paquet 2005) using methods described in Smyth and Speed (2003), Smyth (2004), Smyth et al. (2005), and Dudoit and Yang (2002). Within-array data were normalized by 2D spatial loess and print-tip loess detrending procedure. Data were then scaled to have the same median absolute deviation across arrays. Nonspecific filtering was applied to reduce false discovery rate by removing invalid and low intensity genes. Intensity filtering was done with the genefilter package to remove genes whose A values were smaller than 7 in at least 75% of the samples. Linear models were fitted to the normalized data using duplicate correlations, and empirical Bayes analysis was used to compute moderated t statistics, which were then used to obtain P values. For multiple testing, the P value adjustment method of Benjamini and Hochberg (1995) was applied to control the false discovery rate (i.e., expected proportion of truly nondifferentially expressed genes among the rejected hypotheses). An adjusted P value cutoff of 0.01 was used to generate differentially expressed gene lists. Differentially expressed genes were chosen if, in addition to displaying an adjusted P value of ≤ 0.01, the fold change was > 1.5 or < 0.67. Standard annotation for the genes on the array was provided by TIGR. Differentially expressed genes were also manually categorized according to MIPS functional categories (FunCat; mips.gsf.de/projects/funcat), using the FunCat assignments of highly similar sequences from other species as guides.

Confirmation of the microarray data was performed by

Table 1. Primer pairs selected for real time quantitative reverse transcriptase polymerase chain reaction (RT-PCR).

Clone	5′–3′ sequence	3′–5′ sequence
STMCX33	CACATATCGATTCCCTATTTTTTGG	CCATCATCCGACTCCGACTT
STMDJ96	CAAACAAAACCCCACAAACTACTTCACT	GCTGTGGCATTGACACTTGACACTT
STMCL31	CCAATAACAGATCAAGCCATAAGTGA	GCTCCAGAACAACCCAAAT
STMFB59	GGAACTGTTGGTTCTAGTGATGATTC	TGTAGCACATATGTCCAGTTTCATGT
STMCO50	CATTGTTTTCTTTCTTCTTGCAACTTCCT	GACTTCTGGTCCATCACTTTCTTTTCG
STMEP88	GGCAACTTTCATGCGTCAAA	GCACTAATTCGCTGATGAAATTGT
18S rRNA*	GGGCATTCGTATTTCATAGTCAGAG	CGGTTCTTGATTAATGAAAACATCCT

*GenBank accession number X67238.

real time quantitative RT-PCR using primers derived from the sequence of the Current TC of the clones available at www.tigr.org/tdb/potato/search/potato_search_basic.shtml.

Real time quantitative reverse transcriptase polymerase chain reaction

TaqMan reverse transcription reagents (Applied Biosystems, Foster City, Calif.) were used to synthesize cDNA. Reaction conditions were 1× TaqMan RT buffer, 5.5 mmol·L^{-1} $MgCl_2$, 500 μmol·L^{-1} deoxynucleotide triphosphates (dNTPs), 2.5 μmol·L^{-1} random hexamers, 0.4 U·μL^{-1} RNase inhibitor, 1.25 U·μL^{-1} multiscribe reverse transcriptase; 25 °C for 10 min, 48 °C for 30 min, and 95 °C for 5 min.

Real time quantitative PCR was performed using 7500 Real-Time PCR System (Applied Biosystems) with the following parameters: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min. Power SYBR Green PCR Master Mix (Applied Biosystems) was used in a final reaction volume of 25 μL. Target gene primers were used at a final concentration of 900 nmol·L^{-1} and 18S ribosomal endogenous control primers at 100 nmol·L^{-1}.

To utilize the comparative C_T method of relative quantitation of gene expression, validation experiments were performed on all target gene primers. Primer pairs used for this work are listed in Table 1. The primers used for 18S rRNA were taken from Nicot et al. (2005). All target gene primers had an amplification efficiency similar to the 18S amplicon (absolute value of the slope of ΔC_T (target gene-18S) versus log input RNA were all < –0.1). Dissociation curves were performed for all primer pairs to check specificity of primers for the target gene. 18S rRNA was used as an endogenous control. Fold change levels of gene expression were expressed as RQ (relative quantitation) values using a "calibrator" sample (RNA from control leaf at zero time) as a reference using Sequence Detection Software version 1.4 (Applied Biosystems).

Results

Two different treatments were designed to explore the effect of CPB larvae feeding on the potato transcriptome. In the first treatment, leaves were exposed to the larvae for 1 h: after 1 h, the larvae were removed, and the infested leaves were harvested 3 h later. In the second treatment, leaves were exposed to larvae continuously for 4 h, then harvested immediately thereafter. The former treatment resulted in the consumption of approximately 10% of the exposed leaf area, while the latter treatment resulted in about threefold greater damage. While both of these treatments represent relatively short-term responses of potato to CPB feeding, the design of the two treatments permits examination of different aspects of the defense response. The continuous-feeding treatment exposes the plant to persistent injury, and as such provides a snapshot of the local defense against classic, relatively severe herbivory. In contrast, the treatment in which leaves were exposed to a shorter interval of herbivory followed by a recovery period provides an opportunity to identify genes induced locally as a means of protection against both the present and any potential future herbivore attack, as well as to perhaps view modes of damage repair following herbivore attack.

Two-color microarray analyses were performed to compare locally damaged leaves from each of these two treatments versus analogous leaves from uninfested control plants. Supplementary data,[2] Tables 1 and 2, list genes that were determined by microarray analyses to be differentially expressed in CPB-infested leaves relative to control leaves after 1 h of CPB feeding followed by 3 h recovery (1F/3R) or 4 h of continuous CPB feeding (4F/0R), respectively. Following statistical analyses of the microarray data sets in R, differentially expressed (DE) genes were determined on the basis of an adjusted P value of less than 0.01, in addition to a fold change in average signal intensity greater than 1.5 (in the case of induced genes) or less than 0.67 (in the case of repressed genes). Gene annotations are based on sequence similarity to the nonredundant database at the National Center for Biotechnology Information, queried using BLASTX (Altschul et al. 1997). In addition to these annotations, genes were classified into functional categories according to the MIPS Functional Catalog scheme version 2.1 (Ruepp et al. 2004; mips.gsf.de/projects/funcat), with minor modification.

A total of 268 genes were found to be significantly DE in the 1F/3R treatment. Of the 268 DE genes, 235 (88%) from the 1F/3R treatment were induced by herbivory (see supplementary data,[2] Table 1). Within this list of induced genes, the most represented functional categories include secondary metabolism (19.5%), stress response (10.2%), signal transduction (8.1%), carbon-compound and carbohydrate metabolism (6.8%), and biogenesis of cellular components

[2] Supplementary data for this article are available on the journal Web site (http://botany.nrc.ca) or may be purchased from the Depository of Unpublished Data, Document Delivery, CISTI, National Research Council Canada, Building M-55, 1200 Montreal Road, Ottawa, ON K1A 0R6, Canada. DUD 3799. For more information on obtaining material refer to http://cisti-icist.nrc-cnrc.gc.ca/cms/unpub_e.html.

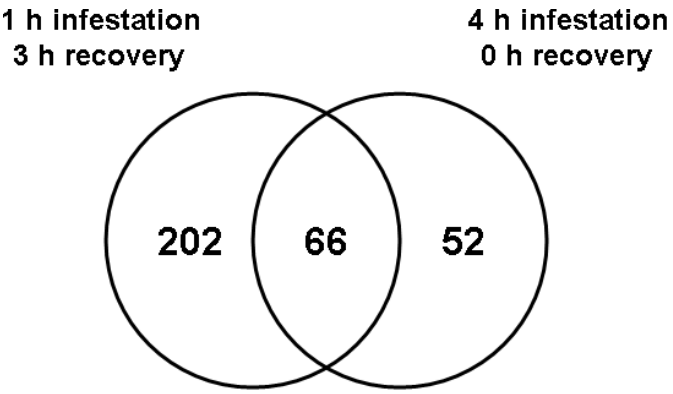
Table 2. Functional categorization of differentially expressed (DE) genes in Colorado potatoe beetle (CPB)-infested leaves according to the MIPS classification scheme.

	Number of induced genes		Proportion of induced genes		
MIPS functional category	1F/3R	4F/0R	1F/3R	4F/0R	Ratio
Secondary metabolism	46	13	0.195	0.118	1.65
Stress response	24	14	0.102	0.127	0.80
Signal transduction	19	6	0.081	0.055	1.48
C-compound and carbohydrate metabolism	16	4	0.068	0.036	1.86
Biogenesis of cellular components	14	7	0.059	0.064	0.93
Hormone metabolism	11	4	0.047	0.036	1.28
Redox	9	1	0.038	0.009	4.19
Transport	9	5	0.038	0.045	0.84
Protein fate	7	1	0.030	0.009	3.26
Transcriptional control	6	7	0.025	0.064	0.40
Lipid and fatty acid metabolism	5	3	0.021	0.027	0.78
Energy	5	1	0.021	0.009	2.33
Amino acid metabolism	3	0	0.013	0.000	n/a
Detoxification	3	1	0.013	0.009	1.40
Transcription	3	2	0.013	0.018	0.70
Protein synthesis	3	1	0.013	0.009	1.40
Protein with binding function	3	1	0.013	0.009	1.40
Unassigned	50	39	0.212	0.355	0.60
Total induced genes	236	110			

(principally cell walls) (5.9%) (Table 2). Table 3 outlines a number of DE genes included in the secondary metabolism category that encode enzymes of defensive chemistry, including volatiles, terpenoids, alkaloids, and phenylpropanoids. The stress response category also includes several genes well documented as responsive to herbivory and (or) pathogens, such as proteinase inhibitors. Not unexpectedly, a number of genes encoding proteins of unknown function were also induced (21.2%), in part reflecting the lack of complete coding sequence information for many genes represented by the cDNAs on the array. Metabolism figures prominently in the defense response, with more than a third (35.8%) of the genes induced by 1F/3R encoding proteins implicated in primary or secondary metabolism. A total of 31 genes were repressed by 1F/3R (see supplementary data,[2] Table 1). The most represented functional categories of proteins encoded by these genes are C-compound and carbohydrate metabolism (15.6%), transcriptional control (12.5%), and unknown function (34.2%).

Interestingly, after 4F/0R, only 118 DE genes were identified, with 110 of these (93%) induced by herbivory (see supplementary data,[2] Table 2). The greatest number of induced genes fell into the functional categories of stress response (12.7%), secondary metabolism (11.8%), biogenesis of cellular components (principally cell walls) (6.4%), and transcriptional control (6.4%) (Table 2). More than a third of the induced genes are of unknown function (35.5%). There are 66 DE genes in common between the two treatments, with 64 of the genes in this common set being induced by herbivory (Fig. 1; supplementary data,[2] Table 3). These induced genes fall mainly into the categories of secondary metabolism (16.7%), stress response (15.2%), biogenesis of cellular components (mainly cell wall) (9.1%), and unknown function (27.3%). Many of the genes that are induced in both treatments represent well-known defense responses, and thus may be considered as a

Fig. 1. Venn diagram illustrating the subset of genes differentially expressed in 1F/3R plants only, 4F/0R plants only, or in both 1F/3R and 4F/0R plants.



"core response". In addition to this shared set of induced genes, the overall pattern of representation by Funcat category (Ruepp et al. 2004) is similar for the two treatments (Table 2). However, a comparison of the induced gene lists for the two treatments reveals differences in the relative proportion of induced genes in specific functional categories: a proportionately greater percentage of genes induced by 1F/3R relative to that induced by 4F/0R fall into C-compound and carbohydrate metabolism (6.8% vs. 3.6%), secondary metabolism (19.5% vs. 11.8%), protein fate (3.0% vs. 0.9%), redox (3.8% vs. 0.9%), and signal transduction (8.1% vs. 5.5%). Table 3 illustrates that many fewer genes involved in secondary metabolism are induced after 4F/0R than after 1F/3R. Notably, no genes implicated in alkaloid or terpenoid biosynthesis are significantly induced in the 4F/0R treatment.

Real time quantitative RT-PCR results of six genes that are differentially expressed by microarray are shown in

Table 3. Colorado potato beetle (CPB) herbivory-induced genes encoding enzymes of secondary metabolism implicated in defensive chemistry.

		Fold change[a]	
Clone	Annotation	1F/3R	4F/0R
Volatiles			
STMCL31	*S*-Adenosyl-L-methionine : salicylic acid carboxyl methyltransferase	3.47	ns
STMIO46	*S*-Adenosyl-L-methionine : carboxyl methyltransferase	1.51	ns
STMEP88	Aromatic amino acid decarboxylase 1A	1.86	5.75
STMIS58	Phenylacetaldehyde synthase	1.50	ns
Alkaloids			
STMCF39	2-Oxoglutarate-dependent dioxygenase	2.08	ns
STMCL06	2-Oxoglutarate-dependent dioxygenase	5.00	ns
STMCM12	2-Oxoglutarate-dependent dioxygenase	4.82	ns
STMCN85	2-Oxoglutarate-dependent dioxygenase	4.10	ns
STMCY27	Deacetylvindoline 4-*O*-acetyltransferase	2.28	ns
STMEH84	Rhamnose:beta-solanine – beta-chaconine rhamnosyltransferase	5.28	ns
STMGI29	2-Oxoglutarate-dependent dioxygenase	5.10	ns
STMGT67	Tropinone reductase I	ns	0.63
Isoprenoids and terpenes			
STMCY65	1-Deoxyxylulose 5-phosphate synthase	1.74	ns
STMIR06	Isoprenyl diphosphate synthases	2.10	ns
STMCQ50	Monoterpene synthase 2	4.95	ns
STMFB69	Sesquiterpene synthase	1.57	ns
STMHI44	Terpene cyclase	1.58	ns
Chorismate			
STMGL51	Phospho-2-dehydro-3-deoxyheptonate aldolase 1	1.77	2.40
STMHN07	3-Dehydroquinate dehydratase – shikimate dehydrogenase isoform 2	1.81	ns
STMEB53	Chorismate mutase	1.68	ns
STMIO04	Chorismate mutase chloroplast (CM1)	1.82	ns
Phenylpropanoids, phenylpropanoid derivatives, and flavonols			
STMGQ39	Phenylalanine ammonia-lyase	ns	2.04
STMCS41	Cinnamic acid 4-hydroxylase	2.10	2.59
STMCN71	4-Coumarate : coenzyme A ligase	1.93	ns
STMCU55	4-Coumarate-CoA ligase-like protein	ns	1.87
STMIM29	4-Coumarate–CoA ligase 2 (4CL 2)	1.72	2.89
STMES07	Hydroxycinnamoyl transferase	1.49	1.64
STMJO36	Caffeic acid *O*-methyltransferase II COMT	1.68	ns
STMEC84	Caffeic acid *O*-methyltransferase II COMT	1.58	ns
STMEI69	Caffeic acid *O*-methyltransferase II COMT	1.63	ns
STMJL95	Caffeoyl-CoA *O*-methyltransferase (CCoAMT)	2.53	ns
STMEZ84	*N*-Hydroxycinnamoyl-CoA : tyramine *N*-hydroxycinnamoyl transferase THT7–8	1.51	4.13
STMIP44	Tyramine hydroxycinnamoyl transferase (THT)	ns	4.01
STMJE63	Tyramine hydroxycinnamoyl transferase	1.71	3.75
STMCQ37	Flavonol synthase	2.01	1.97
Other			
STMCI55	Polyphenol oxidase	1.68	ns
STMCG85	Cytochrome P450, putative	2.32	ns
STMCX10	Cytochrome P450, putative	4.50	4.62
STMDE16	Cytochrome P450, putative	2.15	ns
STMEA65	Cytochrome P450, putative	1.56	ns
STMGB09	Cytochrome P450, putative	2.47	ns
STMGN13	Cytochrome P450, putative	2.46	ns
STMJE59	Cytochrome P450 71D7	2.16	4.12
STMGO18	Dioxygenase	3.26	ns
STMHE18	2OG-Fe(II) oxygenase	0.63	ns
STMIC94	Oxidoreductase	1.54	ns
STMJJ75	Oxidoreductase 2OG-Fe(II) oxygenase	1.62	ns

[a]Fold change is indicated only for genes with adjusted $P \leq 0.01$ in statistical analysis of microarray data; ns, $P > 0.01$.

Fig. 2. Real time quantitative reverse transcriptase polymerase chain reaction (RT-PCR) of genes induced after infestation. Squares represent leaves that were infested for 1 h and harvested at times indicated on the *x* axis. Triangles represent leaves from plants that were infested continuously for the times indicated on the *x* axis. Diamonds represent leaves from uninfested control plants harvested at the times indicated on the *x* axis. RQ, relative quantitation, fold change of the RNA in the sample compared with the calibrator. (A) STMEP88, aromatic amino acid decarboxylase; (B) STMFB59, class IV chitinase; (C) STMDJ96, JAZ1; (D) STMCX33, cysteine protease inhibitor; (E) STMCO50, proteinase inhibitor 1; (F) STMCL31, *S*-adenosyl-L-methionine : salicylic acid carboxyl methyltransferase, SAMT.

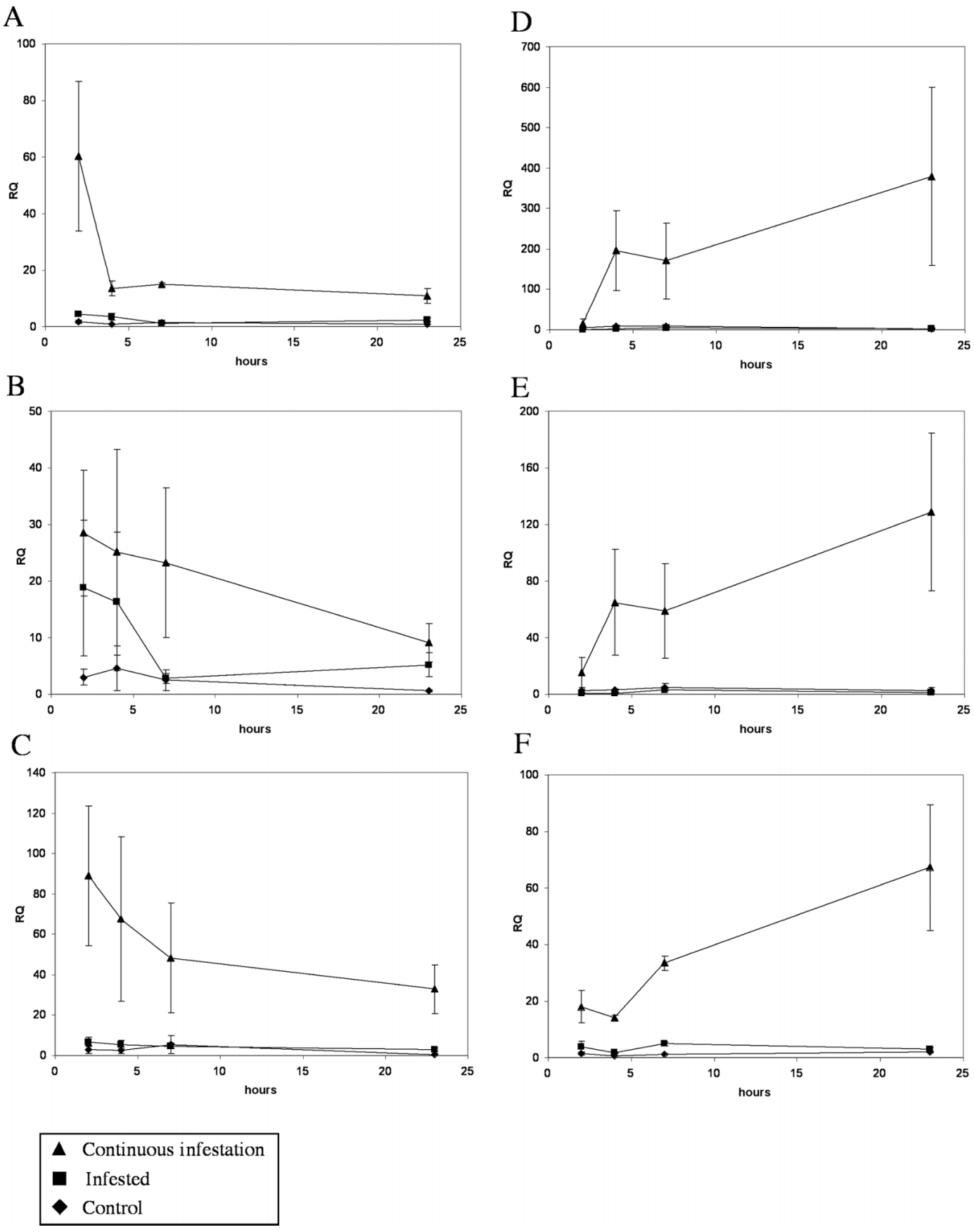
Fig. 2, allowing independent verification of results derived from the microarray data. These genes were selected because they may be important candidates for the direct or indirect defense arsenal against CPB. STMCL31 (*S*-adenosyl-L-methionine : salicylic acid carboxyl methyltransferase, SAMT) and STMDJ96 (jasmonate ZIM domain protein 1) are not present on the 4 h infestation gene list, because the adjusted *P* values are between 0.01 and 0.05, which is higher than our <0.01 cutoff (see supplementary data,[2] Table 2). In agreement with the real time quantitative RT-PCR data, the fold change values of the microarray results for these genes are greater in the 4 h continuous infestation than in the plants with only an initial 1 h infestation. For some genes, the variation within different biological replicates is large, especially during continuous infestation. This is probably due to the plants receiving nonuniform infestation by CPB between different plants even at the same time point. It is interesting that the same samples result in little variation for STMEP88 and STMCL31 compared with the other genes, both of which putatively encode enzymes responsible for the volatile blend attractive to predators of CPB. Roughly the genes can be divided into those in which the controls have about as much transcript as those infested for 1 h, for example, STMCO50 and STMCX33, whereas the remaining four genes contain lower levels of transcripts, in the controls over time, than in the plants infested for 1 h. For STMCO50, STMCL31, and STMCX33 apparently, prolonged infestation enhances expression of these genes.

There is an early peak of transcript for STMFB59, STMDJ96, and STMEP88 (Figs. 2A–2C), while the 23 h continuous infestation resulted in the greatest amount of transcript for STMCL31, STMCX33, and STMCO50 (Figs. 2D–2F). That the different genes had different peak levels of transcript indicates that some respond more quickly to infestation while others build with exposure. In addition, this indicates that the 23 h continuous infestation does not simply result from a steadier and stronger infestation resulting in more infestation-induced transcripts. STMFB59 encodes a putative type IV chitinase, and its transcript level appeared to peak early and decreased after the exposure to CPB. STMCO50 and STMCX33 encode a putative proteinase inhibitor 1 and a putative cysteine protease inhibitor, respectively. The transcript levels barely register when subjected to a 1 h exposure to CPB feeding, but 23 h of continuous infestation results in dramatic levels of these transcripts. Clearly, the expression of transcript for these inhibitors is affected by the continued presence of the insect.

Discussion

Transcriptional profiling by microarray analysis is a powerful tool not only to identify genes previously not known to be associated with a given biological phenomenon, but also to reveal networks of genes that function in a coordinated fashion to drive metabolic pathways or cellular processes. This type of comprehensive profiling approach is ideal for unraveling the many, complex responses of plants to herbivore attack. Microarrays have been used successfully to construct comprehensive portraits of defense responses for a number of herbivore–plant interactions, including those between poplars and forest tent caterpillars (Ralph et al. 2006*a*), spruce and spruce budworm (Ralph et al. 2006*b*), spruce and white pine weevil (Ralph et al. 2006*b*), *Arabidopsis* and cabbage worm (Reymond et al. 2000, 2004), and *Arabidopsis* and Egyptian cotton worm (Reymond et al. 2004). An important outcome that emerges from these studies is that while there are common themes to the defense response mounted by these different species against these different herbivores, there are also species-specific signatures to the defense arsenal, particularly in the defensive chemistry that is invoked to fend off the infesting insect. Recognizing that there may be important aspects of potato–CPB interactions that are unique to this system, or at least underrepresented in other systems, we embarked on an analysis of the transcriptional response of potato to attack by CPB as an important first step in understanding how this economically important crop species defends itself against this devastating pest. The comprehensive studies cited above illustrate that many genes important to a plant's defense response display different dynamics of transcriptional activation. In other words, defense-associated genes are transcriptionaly upregulated at different time points upon infestation, and the amplitude and timeframe of upregulation can vary considerably. Accordingly, we used two different treatments to examine early responses of potato to CPB attack: one in which plants were exposed to CPB for a short interval, then allowed to recover for a period prior to harvest (1F/3R), and the other in which plants were exposed to continuous CPB feeding for a longer period (4F/0R). While the transcript profiling results of these two treatments clearly illustrate different aspects of the defense response, both treatments focus on relatively early responses. Genes that are induced either very early in the defense response in a transient fashion, or genes that are induced only at later phases of the response, may have been missed in this analysis, as would genes induced only systemically rather than locally. Defense responses that do not involve changes in transcript abundance will also not be revealed using a microarray approach.

A conspicuous and perhaps counterintuitive finding of the present study is that a much larger number of genes was significantly upregulated in plants infested with CPB for 1 h, followed by 3 h of recovery prior to sampling than in plants infested with CPB for 4 h continuously prior to sampling. Many of the genes that are significantly upregulated in 1F/3R leaves but not in 4F/0R leaves represent biosynthetic pathways that require significant investment of



carbon resources. We hypothesize that since 4F/0R is a more severe treatment than 1F/3R, 4F/0R has a more negative impact on photosynthetic carbon gain than does 1F/3R. In comparison, 1F/3R plants are not actually under attack at the point of sampling, but rather are in recovery mode. Consequently, we speculate that the 1F/3R plants are able to redirect a greater proportion of carbon resources to defense strategies in locally infested leaves than 4F/0R plants at the time point that we chose for sampling. Although we did not see repression of photosynthetic genes in our analyses, this does not preclude a downregulation of photosynthetic electron transport and (or) the Calvin cycle, particularly in the short time frames that were used for this study. It has been noted in previous studies that genes involved in photosynthesis are repressed by infestation (Hermsmeier et al. 2001; Ralph et al. 2006*a*, 2006*b*), but in these studies, plants exhibiting this response were sampled at least 24 h after initiation of the feeding experiment. In spruce subjected to budworm feeding for 3 or 52 h, for example, changes in photosynthetic genes were only noted at the later time point (Ralph et al. 2006*b*). Furthermore, when potato plants were subjected to wounding and CPB regurgitant, a reduction in genes involved in photosynthesis and carbon metabolism was noted (Lawrence et al. 2008). While further experimentation would be required to test this hypothesis, it is the most congruent explanation of the observations.

Secondary metabolism

There is an enormous number and considerable diversity in the range of natural products (products of secondary metabolism) synthesized by plants. Many of these compounds are thought to play a role in the plants' defense arsenal. Genes involved in secondary metabolism were among the most prominent in the list of upregulated genes of infested plants, illustrating the importance of defensive chemistry in the response of potato to CPB attack. The terpenoid and alkaloid classes of compounds constitute an important component of plant defensive chemistry in several plant species (Dudareva et al. 2006; Keeling and Bohlmann 2006). Several genes encoding enzymes putatively involved in alkaloid and terpenoid biosynthesis were significantly upregulated in 1F/3R leaves; surprisingly, none of these were significantly upregulated in 4F/0R leaves (Table 3).

Tropane alkaloids have been shown to be induced as part of the defense response in some members of the Solanaceae (Alves et al. 2007). A tropane synthase I-like gene was downregulated in 4F/0R leaves. Tropane synthases catalyze reactions at branchpoints in alkaloid biosynthetic pathways; consequently, downregulation of this enzyme would potentially change the identity and (or) ratio of alkaloids synthesized in CPB-infested plants.

Six genes encoding enzymes putatively involved in terpene synthesis were induced significantly in 1F/3R leaves, but not in 4F/0R leaves. These genes represent early steps of the general terpenoid pathway, rather than branches leading to specific terpenoids. Most of these genes have been implicated in the defense response in other plant–herbivore interactions (Pichersky and Gershenzon 2002), presumably reflecting their role in increasing flux of carbon into terpenoid biosynthesis rather than altering concentrations of specific terpenoids. Interestingly, terpenoids make up a portion of the volatiles created by infestation-induced plants, which could play a part in attraction of predator insects and parasitoids to the infested potato.

Other genes that may play a role in the generation of volatile defensive compounds were also upregulated. A gene encoding aromatic amino acid decarboxylase was induced by infestation, a result verified by real time quantitative RT-PCR (Fig. 2A). This enzyme is involved in the production of phenylalanine-derived volatile compounds important for insect attraction (Tieman et al. 2006). Aromatic amino acid decarboxylase is the enzyme responsible for the precursor of 2-phenylethanol (Tieman et al. 2006). This is a volatile specific to CPB-damaged plants (Schütz et al. 1997), and is found to be particularly attractive to the CPB predator *Perillus bioculatus* (Fabr.) (Weissbecker et al. 1999). Our data suggest that 2-phenylethanol is a volatile produced in response to CPB infestation of potato plants. This plant response is part of a tritrophic interaction, which may indirectly result in plant defense. An additional gene that encodes *S*-adenosyl-L-methionine : salicylic acid carboxyl methyltransferase (SAMT) was also induced. This enzyme is responsible for the conversion of salicylic acid to methyl salicylic acid, a component of a volatile blend produced upon CPB feeding that attracts CPB and its predator *Podisus maculiventris* (Say) (Dickens 1999). We found that a SAMT-like gene is also induced in maize by infestation of beet armyworm (Lawrence and Novak 2004); similarly, a rice SAMT-like gene is induced by wounding and pathogens (Xu et al. 2006). A biochemically and molecularly characterized *S*-adenosyl-L-methionine methyltransferase from *Arabidopsis* that shows activity with both salicylic acid and benzoic acid has also been implicated in plant defense (Chen et al. 2003). This gene, whose expression correlates with methyl salicylate emissions from an attacked plant, is strongly upregulated in response to methyl jasmonate, herbivory, and fungal pathogen elicitors.

Many genes of the phenylpropanoid pathway were induced in the infested leaves (Table 3). The monolignol precursors of lignin biosynthesis are a key product of the phenylpropanoid pathway; lignin is important in cell wall reinforcement. Most of the phenylpropanoid genes upregulated by CPB herbivory catalyze reactions in the upstream portion of the pathway; as such, the presumed increased flux through this pathway may also increase synthesis of other phenylpropanoid-based defensive compounds such as stilbenes.

In addition to the lignin biosynthetic genes, several other genes associated with cell wall biosynthesis and remodeling were also upregulated by CPB infestation, suggesting the importance of cell wall fortification in the defense against herbivores. Several of these genes are enzymes important in the biosynthesis of carbohydrate polymer constituents of cell walls, that is, hemicellulose and pectin. Other cell wall-associated genes upregulated by CPB herbivory include polymer crosslink modification proteins and cell wall structural proteins. Up to five xyloglucan endotransglucosylase-hydrolase (*XTH*) genes were found to be induced by infestation. XTH enzymes function in cell wall modification. Interestingly, *XTH* genes have also been found to be induced by phloem-feeding insects (Heidel and Baldwin 2004; De Vos et al. 2007).

As many as seven different genes potentially encoding cytochrome P450s were induced by herbivory (Table 3). Cytochrome P450s are a large and complex superfamily, with several members of the family having being implicated in defense responses (reviewed by Schuler and Werck-Reichhart 2003). There is remarkable subdiversification within the family: different cytochrome P450s are variously involved in biosynthetic reactions producing such compounds as phenylpropanoids, alkaloids, and terpenoids, as well as in detoxification reactions. Small differences in cytochrome P450 protein sequences can lead to discrimination not only in substrate preference, but also in the products that are produced. As such, cytochrome P450s are a potential key to the diverse array of phytoalexins synthesized in response to herbivory.

Other genes implicated in defense and (or) the general stress response

Numerous pathogen-associated genes were found to be induced by CPB infestation (see supplementary data,[2] Tables 1 and 2). These include transcripts for pathogen-inducible alpha dioxygenase (*PIOX*, Sanz et al. 1998), arachidonic acid-induced *DEA1*(Weyman et al. 2006), harpin-induced proteins (Gopalan et al. 1996), *NtEIG-A1* (Takemoto et al. 2003), jasmonic acid 2 (Tian et al. 2006), and *STS14* (van Eldik et al. 1996). The *PIOX* gene has been found to be inducible by *Manduca sexta* feeding on *Nicotiana attenuata* (Hermsmeier et al. 2001).

Genes encoding chitinases, which are often associated with pathogen attack (Van Loon et al. 2006), are also affected by herbivory (e.g., Philippe and Bohlmann 2007). In this report, the putative class IV chitinase, STMFB59, was rapidly induced by infestation with CPB (Fig. 2B). Since the peritrophic membrane surrounding the insect midgut is made of chitin, this could play a part in defense against insects. In fact, over-expression of a recombinant poplar chitinase in tomato has deleterious effects against CPB (Lawrence and Novak 2006).

Many proteinase inhibitors were found to be induced by CPB infestation (see supplementary data,[2] Tables 1 and 2), which is consistent with other studies of plant–herbivore interactions (Philippe and Bohlmann 2007). Two of these, STMCX33 and STMCO50, encode a putative cysteine protease inhibitor and a putative proteinase inhibitor 1, respectively. The transcripts for both of these genes were dramatically induced by long-term continuous infestation (Figs. 2D and 2E). CPB larvae contain cysteine and aspartate proteinases (Wolfson and Murdock 1987), as well as a serine proteinase (Novillo et al. 1997). Interestingly, CPB exposed to induced potatoes develop proteases with insensitivity to cysteine inhibitors (Bolter and Jongsma 1995), suggesting that a suite of proteinase inhibitors are required for a broad-scale defense against herbivores.

Candidate regulators of the defense response

The analysis identified a number of genes that putatively encode proteins that function in signal transduction and control of gene expression. For example, a number of genes with similarity to transcription factors were differentially expressed in leaves infested by CPB (see supplementary data,[2] Tables 1 and 2). While most of these were upregulated by CPB attack, transcript of four putative transcription factors — including a putative MYB protein — were downregulated. Of particular interest are two genes encoding proteins similar to jasmonate Zim domain protein 1 (JAZ1) of tomato (Thines et al. 2007). Characterization of JAZ proteins in *Arabidopsis* has shown that they act as repressors of the transcription factor MYC2, which activates jasmonate responsive genes (Chini et al. 2007). The oxylipin JA is a small signaling molecule involved in plant defense against herbivory (Howe 2004), and the upregulation of *JAZ*-like genes suggests a role in regulating the defense response of potato against herbivory. Genes putatively involved in the early steps of the synthesis of oxylipins, including JA, were also induced by CPB infestation. Three genes that may encode patatin-like proteins and two 13-lipoxygenases were induced. The patatin-like proteins may have lipid acyl hydrolase (LAH) activity (La Camera et al. 2004). This is the first step in the breakdown of membranes for the production of JA or green leaf volatiles (GLVs), another class of small signaling molecules. Next 13-lipoxygenase converts 18:3 α-linolenic acid or 18:2 linolenic acid. GLVs can be used as cues by natural enemies in an indirect defense response (Matsui 2006). It is noteworthy that later steps in the synthesis of JA, the gene for hydrogen peroxide lyase and allene oxide synthase, were not present on the inducible gene list, although they were present on the array.

Conclusions

We have presented the first examination of transcriptional profiling of potato genes affected by CPB infestation. The two conditions used for this analysis provide different snapshots of the CPB–potato interaction. This is a valuable first step given the agronomic importance of this plant–insect interaction. It also affords the opportunity to clone and characterize promoters from a number of infestation-induced genes. We found both induced and repressed genes due to infestation. While several of the induced genes seemed to be associated with volatile synthesis, this is by no means the entire story. The effect of proteinase inhibitors on CPB defense warrants further investigation.

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